

**United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health Science**

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Title: Determination of Trenbolone By ELISA		
Revision: .00	Replaces: NA	Effective: 11-30-04

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A. INTRODUCTION

1. Theory

Trenbolone acetate is an anabolic steroid with strong androgenic activity.

The microtiter wells are coated with capture antibodies directed against anti-trenbolone antibodies. Standards or sample solution, trenbolone enzyme conjugate and anti-trenbolone antibodies are added. Free and enzyme conjugated trenbolone compete for the antibody binding sites (competitive enzyme immunoassay (EIA)). At the same time, the anti-trenbolone antibodies are also bound by the immobilized capture antibodies. Any unbound enzyme conjugate is then removed in a washing step. Enzyme substrate (urea peroxide) and chromogen (tetramethylbenzidine) are added to the wells and incubated. Bound enzyme conjugate converts the colorless chromogen into a blue product. The addition of the stop solution leads to a color change from blue to yellow. The measurement is made photometrically at 450 nm (optional reference wavelength, 600 nm). The absorption is inversely proportional to the trenbolone concentration in the sample.

2. Applicability

This test is applicable for the qualitative analysis of trenbolone in bovine liver at levels ≥ 5 ppb.

B. EQUIPMENT

Note: Equivalent apparatus and instrumentation may be substituted for the following items.

1. Apparatus

a. RIDASCREEN® Trenbolon Test Kit:

Each kit contains sufficient materials for 96 measurements (including standard analyses). Each test kit contains:

- i. 1 x Microtiter plate with 96 wells (12 strips with 8 removable wells each) coated with capture antibodies.

Note: Keep unused wells together with the drying agent and well sealed in the foil bag. Keep stored at 2 - 8 °C.

- ii. 1 x Conjugate (0.7 mL), peroxidase conjugated trenbolone concentrate, red cap.

Note: The trenbolone enzyme conjugate (bottle with red cap) is provided as a concentrate. Since the diluted enzyme conjugate has a limited stability, only the amount, which actually is needed, should be reconstituted. Before pipetting, the enzyme conjugate should be shaken carefully. For reconstitution, the conjugate concentrate is diluted 1:11 (1+10) in buffer (e. g. 200 μ L conjugate concentrate + 2 mL buffer, sufficient for 4 microtiter strips).

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- iii. 1 x Anti-trenbolone antibody (0.7 mL), concentrate, black cap.

The anti-trenbolone antibody (bottle with black cap) is provided as a concentrate. Since the diluted antibody solution has a limited stability, only the amount, which actually is needed, should be reconstituted. Before pipetting, the antibody concentrate should be shaken carefully. For reconstitution, the antibody concentrate is diluted 1:11 (1+10) in buffer (e. g. 200 µL antibody concentrate + 2 mL buffer, sufficient for 4 microtiter strips).

- iv. 1 x Substrate (7 mL), contains urea peroxide, green cap.

- v. 1 x Chromogen (7 mL) contains tetramethylbenzidine, blue cap.

The colorless chromogen is light sensitive, therefore, avoid exposure to direct light. Any coloration of the chromogen solution is indicative of deterioration and the reagent should be discarded.

- vi. 1 x Stop solution (14 mL) contains 1 N sulfuric acid, yellow cap.

- vii. 1 x Buffer (25 mL), Conjugate and antibody dilution buffer.

Note: Store the kit at 2 - 8 °C. Do not freeze. Bring all reagents to room temperature (20 - 25 °C) before use. Return all reagents to 2 - 8 °C. immediately after use.

- b. Incubator - Labline, model 100.

- c. Evaporator - Fisher sample concentrator, model 100.

- d. Homogenizer - Omni International 2000.

- e. Scintillation vials - VWR, borosilicate glass with screw cap, 66022-004.

- f. Micropipettes - 10 -100 µL, P1000, multichannel pipetter 8 spot 50 - 300 µL.

- g. C18 column - RIDA®, R-Biopharm AG, Art. No. R2002.

- h. Centrifuge tubes - 15 mL, Falcon.

- i. Disposable culture tubes - borosilicate glass, 16 x 125 mm, Fisher brand.

2. Instrumentation

Microtiter plate spectrophotometer - 450 nm.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted for the following items.

1. Reagents

- a. Methanol - Mallinckrodt UltimAR, H488-10.

- b. Tert-butylmethylether - Sigma Aldrich, HPLC grade, 293210-2L.

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- c. NaOH pellets - J.T. Baker, 500g, 3722-11.
- d. Glacial Acetic Acid (CH₃COOH) - J.T. Baker, 500 mL, 9507-00.
- e. Glucuronidase/arylsulfatase of *Helix pomatia* - Merck Art. No. 4114.
- f. Ethanol - E.M. Science.

2. Solutions

- a. 20% acetic acid:
Add 20 mL of glacial acetic acid to a 100 mL volumetric flask. Dilute to volume with deionized water.
- b. 500 mM sodium acetate buffer, pH 4.8:
Add 41 g sodium acetate (CH₃COONa) to a 800 mL beaker add 700 mL of deionized water Adjust to pH 4.8 with 20% acetic acid. Transfer to a 1000 mL volumetric flask and dilute to volume with deionized water.
- c. 20 mM PBS-buffer, pH 7.2:
Add 0.55 g NaH₂PO₄ · H₂O + 2.85 g Na₂HPO₄ · 2 H₂O + 9.0 g NaCl to a 1000 mL volumetric flask. Dilute to volume with deionized water.
- d. 80% Methanol:
Add 800 mL of Methanol to a 1000 mL volumetric flask. Dilute to volume with deionized water.
- e. 40% Methanol:
Add 400 mL of Methanol to a 1000 mL volumetric flask. Dilute to volume with deionized water.

D. STANDARDS

1. Source

17 alpha-Trenbolone
RIVM laboratory for Residue analysis,
P.O. Box 1 NL 3720 BA Bilthoven, The Netherlands.

2. Preparation

- a. Trenbolone stock solution (100 µg/mL):

Note: Allow the ampoules to warm to room temperature before opening. Place them in the dark at ambient temperature for at least 30 minutes.

- i. Open the ampoule by carefully breaking the neck. Pipette 1.0 mL of ethanol into the ampoule. Close the ampoule carefully with film (to avoid evaporation

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during the following steps).

- ii. Vortex the ampoule for at least 1 minute.
- iii. Place the ampoule in an ultrasonic water bath for at least 5 minutes.
- iv. Prepare the standard solution.

b. Trenbolone working solution (500 ng/mL):

Pipette 50 μ L of the stock solution into a 10 mL volumetric flask and dilute to volume with methanol.

c. Fortification Solution (50 ng/mL):

Pipette 1 mL of the working solution into a 10 mL volumetric flask and dilute to volume with methanol.

d. Spotting standard #1 (10 ppb):

Pipette 20 μ L of the fortification solution into a 10 mL volumetric flask and dilute to volume with methanol.

e. Spotting standard #2 (5 ppb):

Pipette 5 mL of spotting standard #1 into a 10 mL volumetric flask and dilute to volume with methanol.

f. Spotting standard #3 (0 ppb):

Use 40% methanol for this solution.

3. Storage and Stability

- a. The stock solutions are stable for 6 months if kept in freezer.
- b. The working standard solutions are stable for up to 2 months if kept in freezer.
- c. Spotting standards are stable for up to 2 months if kept in freezer.

E. SAMPLE PREPARATION

Homogenize liver or muscle samples and freeze if samples will not be started the same day as prepared.

F. ANALYTICAL PROCEDURE

1. Extraction Procedure

- a. Weigh 1 g of ground liver sample into a 15 mL Falcon tube. Weigh 2 blank samples to be used as a blank and fortified control. For fortified control, add 100 μ L of 50 ng/mL fortification solution for a concentration of 5 ppb.
- b. Add 2 mL of 0.5 M sodium acetate buffer, pH 4.8, to each sample and homogenize.

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- c. Add 8 μ L of glucuronidase/arylsulfatase of *Helix pomatia*.
- d. For hydrolysis, cap tube and incubate the solution for 3 h at $37 \pm 1^\circ \text{C}$, or alternatively over night at room temperature.
- e. Add 5 mL of tert-butylmethylether to the hydrolyzed product in the 15 mL Falcon tube and shake vigorously for 30 - 60 min.
- f. Centrifuge: Approximately 10 min / \cong 3000 g / \cong 10 - 15 $^\circ\text{C}$.
- g. Transfer the supernatant to a scintillation vial.
- h. Repeat the extraction with another 5 mL of tert-butylmethylether.
- i. Reduce the combined ether layers to dryness using concentrator and then dissolve with 1 mL of methanol (80%) by shaking on a shaker for 15 min.
- j. Dilute the methanolic solution with 2 mL of 20 mM PBS-buffer.
- k. Purify the hydrolysis product by means of RIDA® C18 column in the following manner (flow rate: approximately 1 drop per sec):
 - i. Rinse the column with 3 mL methanol (100%).
 - ii. Equilibrate the column with 2 mL of 20 mM PBS-buffer, pH 7.2.
 - iii. Apply sample (volume after hydrolyzation or extraction as described).
 - iv. Rinse the column with 2 mL 40% methanol. Press out residues of rinse-solution, dry column for 3 min by pressing air or N_2 through it.
 - v. Elute slowly with 1 mL 80% methanol (flow rate: approximately 15 drops per min).
 - vi. Dilute the eluate 1:2 (1+1) with deionized water.
 - vii. Use 20 μ L per well in the test.

2. Test Procedure

Note: Do not allow microwells to dry between working steps.

- a. Insert a sufficient number of wells into the microwell holder for all standards and samples to be run in duplicate. Record the standard and sample positions. The 5 ppb fortified control should be randomly placed into 6 wells throughout the plate.
- b. Add 20 μ L of each spotting standard solution or prepared sample to separate duplicate wells and add 50 μ L of diluted enzyme conjugate to each well.
- c. Add 50 μ L of the diluted anti-trenbolone antibody solution to each well. Mix gently by rocking the plate manually several times and incubate 2 h at room temperature (20 - 25 $^\circ\text{C}$).

Note: Avoid direct sunlight during all incubations. Covering the microtiter plates is recommended.

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- d. Pour the liquid out of the wells and tap the microwell holder upside down vigorously (three times in a row) against absorbent paper to ensure complete removal of liquid from the wells. Fill all wells with 250 μ L of deionized water and pour out the liquid again. Repeat two more times.
- e. Immediately add 50 μ L of substrate and 50 μ L of chromogen to each well. Mix gently by rocking the plate manually several times and incubate for 30 min at room temperature (20 - 25 $^{\circ}$ C) in the dark.
- f. Add 100 μ L of the stop solution to each well. Mix gently by rocking the plate manually and measure the absorbance at 450 nm against an air blank. Read within 60 minutes after addition of stop solution.

3. Instrumental Settings

Set photometer at 450 nm.

G. CALCULATIONS

1. Evaluate sample results based on absorbance values for fortified control tissue. Trenbolone concentration is inversely related to intensities of yellow color.
2. Calculate the mean and standard deviation (SD) for the absorbance readings of the six 5 ppb control replicates. Use these to determine a decision level (DL) using the formula $DL = \text{Mean} + 3 \cdot SD$. Identify the sample as positive if its average absorbance is less than the decision level.

H. SAFETY PRECAUTIONS AND INFORMATION

1. Required Protective Equipment - Lab coat, safety glasses and protective gloves.

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2. Hazards

Procedure Step	Hazard	Recommended Safe Procedures
Stop solution	Fumes are corrosive. Spattering may result in serious eye, skin, and respiratory damage.	Prepare solutions in a well-ventilated area such as a fume hood and dispense using repipettors wherever possible. Wear plastic gloves.

3. Disposal Procedures

Procedure Step	Hazard	Recommended Safe Procedures
Stop solution	See above	Neutralize and flush down the sink. Observe all Federal, state and local environmental laws.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

- a. A plate must meet all of the following criteria:
 - i. The external standard curve has absorbances continuously increasing from the 10.0 ng/mL through each lower concentration standard through the 40% methanol blank spotting solution.
 - ii. The absorbances increase from the 10.0 ppb to the 0 ppb fortified control tissue wells.
 - iii. No false positives for the negative control or false negatives for the positive control.

2. Critical Control Points and Specifications

- a. Do not use RIDASCREEN® Trenbolon kit past the expiration date on the kit label. Dilution or adulteration of these reagents may result in loss of sensitivity.
- b. Do not interchange individual reagents between kits of different lot numbers.
- c. Reproducibility in any EIA is largely dependent upon the consistency with which the

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microwells are washed. Carefully follow the recommended washing sequence as outlined in the EIA test procedure.

- d. Incubations performed at steps 2c and 2e must be carried out at room temperature (20 - 25 °C).

3. Readiness To Perform

a. Familiarization

- i. Phase I: Standards- Duplicate external standard curves on each of 3 consecutive days, which will include the following:
 - (a) 0 ng/mL
 - (b) 5.0 ng/mL
 - (c) 10.0 ng/mL
- ii. Phase II: Fortified samples - Triplicate analyses at 0, 5, and 10 ppb over a period of 3 different days using bovine liver tissue.
Note: Phase I and Phase II may be performed concurrently.
- iii. Check samples for analyst accreditation.
 - (a) 20 unknown beef liver samples. The sample fortifications including the number of blanks are to be blind to the analyst. 6 - 10 of the 20 samples should be blank and the rest fortified at 5 ppb. All samples should be put in duplicate wells. The samples must be randomized throughout the set. An external curve must be run to help monitor plate acceptability.
 - (b) Report analytical findings to the Laboratory Quality Assurance Manager (QAM).
 - (c) Letter from QAM is required to commence official analysis.

b. Acceptability criteria

- i. No false negatives or positives determined for controls.
- ii. Refer to section I.1 above.

4. Intralaboratory Check Samples

a. System, minimum contents.

- i. Frequency: 1 weekly per analyst as samples analyzed.
- ii. Records are to be maintained by the analyst

b. Acceptability criteria.

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Refer to section I.1 above.

If unacceptable values are obtained, then:

- i. Stop all official analyses by that analyst.
- ii. Take corrective action.

5. Sample Acceptability and Stability

- a. Matrix: Beef liver
- b. Sample receipt size: Approximately 200 g.
- c. Sample receipt condition: Frozen
- d. Sample storage:
 - i. Sample: 25 weeks if stored frozen at ≤ -20 °C.
 - ii. Extract:
 - a. Hydrolyzed samples can be stored prior to C18-purification for one or two days at 2 - 8 °C and for two weeks at ≤ -20 °C.
 - b. Eluates in 80% or 40% methanol can be stored well-sealed for two months at ≤ -20 °C or two weeks in the refrigerator.

Note: Frequent thawing and freezing should be avoided.

6. Sample Set

- a. Each sample set must contain:
 - i. Matrix blank
 - ii. Fortified control
 - iii. Samples

7. Sensitivity

- a. Lowest detectable level (LDL): Not determined.
- b. Minimum proficiency level (MPL): 5.0 ppb.

J. WORKSHEET

The worksheet on the following page can be removed for photocopying.

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TRENBOLONE ELISA WORKSHEET

Weighed (Intials/AC/Date)	
Analysis Started(intials/AC/Date)	
Analysis Completed(Initials/AC/Date)	
Reviewed By:(Initials/Date)	

Species	
Set Number	
Reader Wavelength (nm)	
Fortification Standards	
Fortification Standards	

Incubator, AM (Time/Temp.37±1°C)	
Incubator, PM (Time/Temp.37± °C)	
Concentrator(Temp.40 ± 1°C)	
Centrifuge (10-15 °C)Temp	
Room temperature: (20-25 °C)	

Equipment	ID #
Refrigerator	
Freezer	
Balance	
MCP	
Centrifuge	
Concentrator	
C ₁₈ Column	
Optical Density Reader	
Incubator	

Reagents	ID #
0.5 M Sodium Acetate	
Glucuronidase/Arylsulfatase	
tert-Butylmethyl ether	
0.02 M PBS Buffer	
Methanol	
40% Methanol	
80 % Methanol	
ELISA Trenbolone kit	
Anti-Trenbolone enzyme conjugate	
Antibody (1:11)	
Water	

Remarks:

Number	Lab Number	Form Number	1 st Wt. of sample 1.00 ± 0.10 g	Result (+) or (-)
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				

Number	Lab Number	Form Number	1 st Wt. of sample 1.00 ± 0.10 g	Result (+) or (-)
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				
25				
26				
27				
28				

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K. APPENDIX

1. Specificity

The specificity of the RIDASCREEN® Trenbolon test was determined by analyzing the cross-reactivities to corresponding substances.

Cross-reactions:	17β-trenbolone.....	100%
	Trendione.....	100%
	17α-trenbolone.....	approx. 82%
	17β-trenbolone-glucuronide	approx. 82%
	17α-trenbolone-glucuronide.....	approx. 7%
	19-nortestosterone.....	approx. 0.06%
	Testosterone, estradiol, zeranol, DES and Chloramphenicol.....	< 0.01%

2. Reference

RIDASCREEN® Trenbolon, 02-10-28

Approved By:	Date Approved:
Milo Stubban	11-22-04
Tom Mallinson	11-22-04
Terry Dutko	11-19-04
Sher Ali for Jess Rajan	11-22-04
Charles Pixley	11-19-04
Phyllis Sparling	11-19-04

Approvals on file.